Vol. LVII *December, 1929* *No. 6*

BIOLOGICAL BULLETIN

VARIATION OF NORMAL GERM CELLS. STUDIES IN AGGLUTINATION.

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It has been generally assumed that eggs or sperm, freshly shed from different echinid individuals at the height of the breeding season, are in essentially the same physiologic condition. This assumption has led to gross errors, discordant experimental results and unnecessary confusion.

Nearly everyone who has made prolonged experimental studies with echinoderm eggs or sperm has recorded evidences of a disturbing variability, even in freshly shed germ cells. There has been, however, little or no agreement concerning (1) the exact physiologic condition at the time of maturing or shedding, (2) the extent of the change or changes at fertilization; whether these changes are in one direction or cyclical, (3) the effect of such changes upon the behavior of the fertilized eggs, (4) the number and kind of changes, and (5) the underlying causes of these changes.

In respect to sperm it has also been assumed that they are relatively constant when shed.

As early as 1883 Born noted that the eggs in the last part of the egg string (*Bufo cinereus*) developed irregularly or did not develop at all. Koehler (1915) made a similar observation for echinid eggs, *i.e.*, eggs were not in the same physiologic state when shed, for those nearest the exit pores were the oldest. Lillie, R. S. (1908, 1915) observed that the age of the eggs (starfish) was an important factor in their subsequent behavior. He corroborated Gemmill (1900) that the eggs changed with age, improving at first and subsequently deteriorating. Morgan [1]

[1] Reviewed in his "Experimental Embryology" (1927).

noted that the eggs (*S. purpuratus*) showed signs of ageing even when in the ovary, for the jelly layer was reduced as in eggs that were aged for several days in the laboratory.

Marked changes have been noted towards the close of the breeding season. Gemmill (1900) among others noted that at this time the echinid eggs lived a shorter time, polyspermy occurred more and more frequently, fertilization was delayed, and sperm lived a shorter time. Heilbrunn (1915) recorded that the concentration of sperm (*Arbacia*) required to elevate the fertilization membrane was greater towards the close of the breeding season, and that there was progressively greater difficulty of the eggs to elevate the membrane. Hyman (1923) also noted changes in the viscosity of ageing eggs.

Lillie, F. R. (1914) concluded that "the condition of the gonads is the most variable thing in summer sea urchins . . ." and in 1915 concluded that the large variations (*Arbacia*) were due to the use of germ cells after the height of the breeding season that variations prior to this time are due to faulty technique, for with proper precautions "both eggs and sperm are relatively constant."

Goldforb (1917, 1918) studied the variability of eggs of three species of echinoderms, *Toxopneustes*, *Hippanoë*, *Arbacia*, studied the variation in size, color, shape, amount of jelly, rate and manner of membrane formation, rate and numbers of fertilized eggs, rate and regularity of cleavage. These studies were made during three seasons. He concluded that freshly shed eggs at the height of the breeding season were not constant, that they showed wide ranges of variability, and sometimes even extreme states of deterioration. He concluded that varying degrees of overripening occurred prior to shedding.

Just (1919) came to a similar conclusion after studying *Echinarachnius* eggs. He records a wide variation due largely to corresponding degrees of deterioration. He also concluded that "ovary" eggs (of *Asterias* also) were more variable and inferior to shed eggs, due to underripeness. Graves (1928) found that *Cummingia* eggs behaved as echinid eggs described by Goldforb, namely, that deterioration occurred before spawning, with a corresponding wide range in the vitality of the eggs. Gee (1916)

for *Fundulus* eggs, Calkins (1920) for *Uroleptus*, attributed variations to corresponding internal differences.

Other evidence of changes in the egg, prior to shedding, is afforded by the work of Hertwig (1906, 1907), Kuschekewitch (1910), and Riddle (1914), that overripeness of the eggs decreased developmental energy, and increased the proportion of surviving males. Koehler (1915) noted the tendency of overripe germ cells (echinid) to produce more matroclinous larvæ. Hertwig (1890), Grief (1901), Newman (1921), and others attributed the increase in natural parthenogenesis to ageing of eggs. Fuchs (1914) and East (1915, 1917, 1918) recorded the increase in self fertilization with overripeness of the germ cells.

These citations may serve to indicate the background for the present study of the variation of germ cells, and of the subsequent studies of ageing germ cells.

The present study gives evidence of a surprisingly wide range of variation, not only among eggs, but also among sperm, when these germ cells were freshly collected, freshly shed, and freshly tested, at the height of the breeding season. *These observations emphasize the need, in experimental work, of determining by simple tests, the exact physiologic condition of the eggs and of the sperm of each individual.* By selecting germ cells in approximately the same physiologic condition, one may minimize the disconcerting differences in experimental results.

The experiments were performed in 1924 and in 1926 at the Marine Biological Laboratory at Woods Hole. My thanks are due the Directors for the facilities of the Laboratory.

Technique.

Because so much depends upon the exact details of technique, and to avoid repetition in subsequent studies, a concise statement is here given of the experimental procedure. This is primarily the procedure of Lillie, F. R. (1914). *Arbacia punctulata were brought directly from the collecting boat to the laboratory and immediately tested.* Eggs that spontaneously flowed from the aboral openings are called "shed" eggs. When ovaries were removed as intactly and gently as possible, the eggs thus liber-

ated are termed "ovary eggs." [1] Similarly "shed" and "testes" dry sperm were obtained. The germ cells from each individual were kept separately. Eggs were washed in 300 cc. or more sterile sea-water, and the final egg to water volume was usually 1 to 3. The eggs were gently shaken every ten minutes for one hour. The supernatant or "egg water" was removed, *examined for unripe, ripe, and overripe eggs, and for jelly. If present, they were immediately removed.* Their presence would alter the subsequent agglutination reaction. The "egg water," the solutions therefrom, and the dry sperm, were kept under wet cloths to minimize evaporation and to maintain a lower temperature, 20° C.[2] Capillary mouth pipettes were sterilized (against sperm and agglutinins) in a jar of 8,000 cc. tap water, then rinsed in 3,000 cc. sterile sea-water, then used in transferring the "egg water" solution to the sperm suspension on the slide. Each test was made with a different pipette.

After all the "egg water" solutions were made, each with a different pipette, a 1 per cent. sperm suspension was prepared, two measured drops placed on the slide, and the "egg water" solution gently blown under the raised cover slip. The reversible agglutination of the sperm was measured in seconds. A "loud timer" aided in this.

Sea water was carefully collected in glass at the incoming high tide, filtered, and stored in glass for 3 to 10 days before using, and evaporation minimized, by covering with wet cloths. This sterile sea water was used in all experiments at approximately 20° C.

Experimental Error.

The accuracy of the technique was determined in two ways:

1. *Repetition Test.*—The agglutination test was immediately repeated with other samples of the same solution.

2. *Aliquot Part Test.*—Eggs were divided into two equal parts in similar volumes of sea water and similar egg to water dilution. The egg water of each was then tested, one immediately after the other.

[1] The term "ovary eggs" is used by Just (1919) and myself differently, with correspondingly different results. Just cut the ovaries into pieces and presumably liberated many unripe eggs. In my experiments the ovaries are barely injured, with liberation of minimal number of unripe eggs.

[2] Temp. 20°–22° C. in different experiments.

These two tests were made in nearly every experiment. Any variation in these tests was considered a measure of the experimental error.

Out of 19 such tests the agglutination time was exactly the same in 8 tests. In 6 tests there was a difference of but 1 second. In 1 test the difference was 3 seconds and in 1 test the difference was 4 seconds. *The average difference was 1 second, or 4.5 per cent.* This may be considered the experimental error.

Variation in eggs and in sperm from different individuals was then studied.

The Variation in Freshly Shed Eggs from Different Individuals.

Table I gives the observations in 19 experiments, including 58 females tested separately, 2 to 4 in each experiment. These experiments were made from July 1 to 26. In some experiments the egg to water ratios are not the same for all the females. Hence these ratios are given in each instance so that corrections may be made. A fresh sperm suspension was made for testing the egg waters of two to four females of each series. The table gives the observed agglutination time for each egg water, the calculated agglutination time,[1] the difference or variation in seconds and in per cent.

The freshly collected, freshly shed, and freshly tested eggs in each series varied from 2 to 55 seconds calculated time. The variation was least in Experiments 12, 20 and 26, where the different females differed by 2, 2, and 4 seconds respectively. This is within the experimental error.[2] In 10 experiments the variation was 5 to 10 seconds; in 4 experiments the variation was 11 to 20 seconds; in 1 experiment the variation was 30 seconds, and in 1 experiment the variation was 55 seconds. The average variation was 12.0 seconds. The variation in control experiments ranged from 0 to 4 seconds only, with an average of only 1 second.

The minimal variation was 9 per cent. in 1 experiment. In 3 experiments the variation ranged from 16 to 26 per cent., in 4

[1] Calculated for differences in egg to water ratio.

[2] The percentage differences, 9, 19 and 26 per cent., are however much greater than the maximal experimental error.

experiments from 37 to 45 per cent., in 6 experiments from 66 to 87 per cent., in 3 experiments from 100 to 120 per cent., in 1 experiment 400 per cent., and in 1 experiment 1,300 percent. These averaged 142 per cent. The expermental error averaged 4.5 per cent. *The "best" eggs in each day's collecting at the height of the breeding season varied by as much as 1,300 per cent.*

Table I.

Showing Wide Variation in Agglutination Time When "Normal" Eggs from Freshly Collected, Freshly Examined Females are Tested by the Same Sperm Suspension.

Exp. No.[1]	♀ No.	Agglutination Time in Sec.	Egg to Sea-water Ratio.	Egg Water Dilution.	Difference in		[2] Calculated Difference in	
					Seconds.	Per cent.	Seconds.	Per cent.
1	1	55	1 : 3	1/100				
	2	27	1 : 3					
	3	25	1 : 3					
	4	25	1 : 3		30	120		
2	1	12	1 : 4	1/60				
	2	35	1 : 8				55	400
3	1	9	1 : 5	1/60				
	2	12	1 : 6					
	3	23	1 : 2				9	100
4	1	9	1 : 5	1/120				
	2	27	1 : 2				10	110
5	1	13	1 : 8	1/160				
	2	15	1 : 8					
	3	8	1 : 8					
	4	23	1 : 4				7	87
6	1	17	1 : 3	1/80				
	2	13	1 : 3					
	3	22	1 : 3		9	69		
8	1	24	1 : 3	1/160				
	2	14	1 : 3					
	3	23	1 : 3		10	71		
10A	1	0	1 : 6	1/320				
	2	13	1 : 6					
	3	10	1 : 6		13	1,300		

Average experimental error—1 second or 4.5 per cent.

Average variation in "normal" fresh eggs—12.0 seconds or 142 per cent.

[1] These numbers are also the dates in July when each experiment was performed.

[2] To correct for differences in egg to water ratio.

TABLE I.–(Continued)

Exp. No. [1]	♀ No.	Agglutination Time in Sec.	Egg to Sea-water Ratio.	Egg Water Dilution.	Difference in		[2] Calculated Difference in	
					Seconds.	Per cent.	Seconds.	Per cent.
10B	1	23	1 : 3	1/80				
	2	26	1 : 3					
	3	20	1 : 5					
	4	19	1 : 3				7	37
11	1	13	1 : 3	1/320				
	2	16	1 : 3					
	3	14	1 : 5					
	4	15	1 : 3				6	45
12	1	12	1 : 6	1/320				
	2	13	1 : 6					
	3	11	1 : 6					
	4	15	1 : 4				2	19
14	1	29	1 : 2	1/320				
	2	21	1 : 2		8	39		
19	1	50	1 : 3	1/320				
	2	30	1 : 3		20	66		
20	1	23	1 : 5	1/320				
	2	30	1 : 3				2	9
22	1	21	1 : 3	1/300				
	2	26	1 : 3					
	3	18	1 : 3		8	44		
23	1	19	1 : 3	1/300				
	2	18	1 : 2					
	3	19	1 : 3				6	16
24	1	17	1 : 3	1/300				
	2	29	1 : 3					
	3	28	1 : 3		12	70		
25	1	15	1 : 3	1/300				
	2	16	1 : 3					
	3	22	1 : 4					
	4	16	1 : 3				11	77
26	1	13	1 : 2	1/300				
	2	15	1 : 3					
	3	10	1 : 5				4	26

Examination in detail of a few examples may make more clear this extraordinary range in variability. In Experiment I the agglutination was 25 seconds for each of 2 females, 27 seconds and 55 seconds for the other two females with the same egg to water volumes, the same dilution of egg waters, the same sperm

[1] These numbers are also the dates in July when each experiment was performed.

[2] To correct for differences in egg to water ratio.

suspension. Lillie (1914) showed that a more exact procedure consists in finding a dilution of egg water in which the agglutination time is about 8 seconds. When higher concentrations of egg water are used, the agglutination values are not so exact. This is admitted. The necessity for rapid tests with the different suspensions and solutions necessitated the use of a low concentration of egg water but not necessarily the lowest that would give an 8 second agglutination. The fact that the same concentrations were used throughout makes the results comparable, and the error too small to materially affect the results.

In Experiment 8 the agglutination time for the eggs of the 3 females was 24, 14, and 23 seconds respectively. In Experiment 10*A* one female registered 13 seconds, another 10 seconds, *and a third did not agglutinate at all.* In Experiment 22, the values were 18, 21, and 26 seconds respectively.

It should be recalled that this variation occurred in freshly collected urchins, the "best" of the day's collection, at the height of the breeding season, and that the eggs and sperm were freshly shed, immediately after arrival from the collecting boat. These are "normal" eggs. These should register the minimal variation. They actually register a variation from 2 to 1,300 per cent.

In Experiment 19, with a 1/320 egg water dilution, the agglutination time of the eggs of female 1 was 50 seconds. Under exactly the same circumstances the eggs of female 2 registered only 30 seconds. I do not interpret this to mean *that the eggs of female 2 were in the same degree of ripeness as those of female 1, secreting only 3/5 as much agglutinin as female 1.* My interpretation is that in addition to an uncalculable but relatively small genetic difference in agglutinin production [1] *the large difference is due to the greater overripening of the eggs of female 2 prior to shedding.* The evidence in support of this interpretation will be given later. In Experiment 10*A*, with egg water dilutions the same, female 2 registered 13 second agglutination. These fresh "normal" eggs were by a variety of tests shown to be in relatively poor condition, *i.e.*, overripe when shed. The eggs of female 3

[1] Loeb and Chamberlain (1915) measured differences in enzyme content of eggs (*Arbacia*) but whether these differences were genetic, as assumed, or due to ageing, is not clear.

were in still poorer condition, *i.e.*, more overripe, registering only 10 second agglutination. The eggs of equally fresh "normal" eggs of female 1 were extremely deteriorated, giving rise to no agglutination at all.

A much larger number of experiments were made than those listed in Table I. The 19 listed are one series representative of the unexpected, consistent and large variation in freshly shed or "normal" eggs of *Arbacia*.

It might be objected that the calculated differences, to correct for differences in egg to water ratios, are only approximate. But in the experiments where no such calculation was necessary, because all egg to water ratios were the same, the variation was practically as large, namely, 6, 6, 19, 23, 30, 37, 39, 44, 66, 69, 70, 71, 110, 130 per cent.

This extraordinarily large variation in agglutination time closely corresponds with equally large variations in size, color, shape of eggs, thickness of jelly layer, fertilizability, rate of membrane formation, rate, regularity, and per cent. of cleavage. These latter variations have been shown (Goldforb, '18*a*, 18*b*) to represent corresponding degrees of ageing or deterioration. And it is presumptive and later will be demonstrated in detail that the differences in agglutination time also measure degrees of deterioration.

Variation in Freshly Shed Sperm from Different Individuals.

It is concluded that at the height of the breeding season (1) the egg is the actively changing cell, secreting varying amounts of agglutinin; (2) that agglutination time is proportional to the quantity of agglutinin thus liberated. The question arose whether the sperm is a biologic constant, or varies as the egg does.

The following experiments (with those in subsequent studies) demonstrate that *freshly shed sperm from different males are just as widely variable as freshly shed eggs.*

Some typical experiments are brought together in Table II. In Experiment 9, for example, samples of the egg water of female 1 were tested separately by freshly prepared sperm suspensions of

three males. The agglutination was 10, 0, and 9 seconds respectively. When fresh suspensions of the same three males were tested with the egg water of female 2, the results were quite different, namely 17, 0, and 13 seconds, respectively. The 0 denotes no agglutination. Fresh suspensions from the same three males against egg water from female 3 gave even larger agglutination values, namely, 23, 0, and 13 seconds respectively.

Table II.

Showing Wide Variation in Agglutination Time When "Normal" *i.e.* Freshly Shed Sperm from Different Males are Tested by the Same Egg Water.

Exp. No.[1]	♀ No.	♂ 1	♂ 2	♂ 3	♂ 4	Difference in	
						Seconds.	Per cent.
9	1	10	0	9		10	1,000
	2	17	0	13		17	1,700
	3	23	0	13		23	2,300
Aver.		17	0	11			
10	1	15	33			18	120
	2	7	22			15	214
	3	12	17			5	41
13	1	15	14	12		3	25
	2	13	8	12		5	62
	3	13	13	10		3	30
	4	13	11	18		8	63
11	1	12	0	12	9	12	1,200
	2	11	0	16	15	16	1,600
	3	25	0	33	9	33	3,300
	4	10	6	18	15	12	200
	5	17	0	22	13	22	2,200
Aver.		15.0	1.2	20.2	12.2		

[1] These numbers are also the dates in July when each experiment was performed.

It should be noted that the *sperm of male 1 gave consistently the longest agglutinations, with all three females. Male 3 gave intermediate values. Male 2, though freshly shed and "normal", did not agglutinate in any egg water.* The average agglutination time for male 1 was 17 seconds, for male 3, 11 seconds, for male 2, none. Male 1 gave 54 per cent. longer agglutination reactions than male 3, and 1,700 per cent. longer than male 2. If male 2,

whose sperm were not agglutinated in any of the tests, be omitted, the variation is 1, 4, and 10 seconds, or 11, 30, and 77 per cent.

It is also possible by these tests to pick out which eggs are most potent. Female 3 gave the longest agglutination reactions. Female 1 gave the briefest and female 2 intermediate values.

A given sperm suspension gave different values with eggs from different females. Likewise eggs from one female gave different values with sperm from other males. But eggs or sperm of a given individual gave the same relative values with other germ cells.

In Experiment No. 9 an extraordinarily wide difference in agglutinability occurred in the sperms of the three males. *The variation is as great as among eggs from different females. Agglutination time appears to be dependent upon the physiologic condition of the eggs as well as upon the condition of the sperm at the time of testing.* The next study will consider this in detail.

Other experiments gave essentially similar results. In Experiment 10, 2 males were tested separately against 3 females. Male 2 gave consistently longer agglutinations than male 3, namely, 41, 120, and 214 per cent. respectively. In Experiment 13 the sperm of 3 males were tested separately against the eggs from 4 females. The agglutination time varied by 25, 30, 62, and 63 per cent. respectively. In Experiment No. 11, 4 males were tested against 5 females. Male 3 gave consistently longest agglutination values, with an average of 20.2 seconds. Male 1 averaged 15.0 seconds, male 4 averaged 12.2 seconds, and male 2 gave an average of only 1.2 seconds. This male gave no agglutination in 4 out of 5 females. If male 2 be ignored, the difference in agglutinability of these males was 33, 45, 69, 80, and 266 per cent. If male 2 be included, the differences were 1,200, 1,600, 3,300, 200, and 2,200 per cent. The dry sperm of these 4 males had been recorded at the time of shedding as follows: No. 3 best, No. 1 good, No. 4 poor. Male 2 was not recorded. This is in very close agreement with their agglutinability.

The results are unmistakable. The sperm that gives high agglutination values with 1 female tends to give high, though not the same, values with other females. Vice versa, sperm

that gives low agglutination values with one female gives consistently low but not necessarily the same values with other females. The differences with a given sperm are due primarily to differences in the physiologic condition of the eggs of the different females. The reverse is also true, *i.e.*, when a given egg water is tested by different males, the differences observed denote primarily differences in the physiologic condition of the different sperms.

It is evident that the *agglutination time of freshly shed "normal" germ cells is dependent not only upon the condition of the eggs but also upon the condition or agglutinability of the sperm.* Not only do freshly shed eggs from different individuals vary very widely in their ability to agglutinate a given sperm, but a given freshly shed sperm varies as widely in agglutinability, with eggs from different females.

The cause or causes of this variability in sperm will be discussed in the next study.

Variability of Shed Versus Ovary Eggs.

It seemed worth while to compare the agglutination time of "shed" versus "ovary" eggs. It should be recalled that these "ovary eggs" come from females whose *ovaries were removed as intactly and as gently as possible.* These ovary eggs include the minimum of unripe eggs. Such ovary eggs will differ therefore from those described by Just (1919) in which ovaries were cut into pieces, thus liberating many unripe eggs.

For purposes of comparison all experiments, in which the same egg water concentration was used, are brought together in Table III. The eggs of all females in an experiment were tested by the same sperm in fresh suspensions.

Table III indicates that "ovary" eggs tended to give longer agglutination values and a wider range of variability than shed eggs. In the 8 experiments involving 22 females, the "ovary" eggs in each series varied by 1, 4, 5, 7, 8, 8, 12, and 20 seconds respectively. The average was 8.1 seconds. In the 6 experiments including 20 females, the "shed" eggs in each series varied by 1, 1, 3, 4, 6, and 13 seconds. The average was 4.6 seconds. (If female 1 in Experiment No. 9 be omitted, because

no agglutination occurred, the variation among the "shed" eggs would be 1, 1, 3, 3, 4, and 6 seconds, or 3 seconds average.) Experiment 2 is especially interesting because the shed and the ovary eggs were tested by samples of the same sperm. The shed eggs gave 11 and 12 seconds respectively, while the ovary eggs in the same egg water concentration gave much longer values, *viz.*, 15 and 35 seconds respectively.

Table III.

Comparison of "Shed" and "Ovary" Eggs in 1/320 Egg Water Dilution. Each Experiment Tested by the Same Sperm Suspension. Figures Denote Agglutination Time in Seconds.

Exp. No.[1]	Shed Eggs.				Variation in Sec.	Aver. Aggl. Time.	Ovary Eggs.				Variation in Sec.	Aver. Aggl. Time.
	♀ 1	♀ 2	♀ 3	♀ 4			♀ 1	♀ 2	♀ 3	♀ 4		
2	11	12			1	11.5	15	35			20	25
5							8	9	6	13	5	9
9	0	13	10		13	7.7						
10	12	13	11	15	4	12.7						
11	13	16	14	15	3	14.5						
12	14	9	15	12	6	12.5						
14							29	21			8	25
19							28	27			1	27.5
20							23	30			7	26.5
22							22	26	18		8	22
23	20	19	20		1	14.7						
24							17	29	29		12	25
25							16	16	22	16	4	17.5
Average					4.6	13.2					8.1	20.7

[1] Numbers correspond with dates in July when experiment was performed.

The larger variability among "ovary" than among "shed" eggs is attributed to the presence of a larger proportion of overripe eggs. This will be discussed in the next study. More intensive study needs to be made of "ovary" eggs, with a definite knowledge of the relative numbers of unripe, ripe, and overripe eggs, and the degree of overripeness.

Seasonal Variation.

In the first half of July, 1926, nearly all the *Arbacia* gave numerous shed eggs. In the second half of the month there were very much fewer shed eggs and more "ovary" eggs. The

same observations were made in July, 1924. The eggs in the first half of the month were mostly "good" ones, *i.e.*, in good physiologic condition, while those in the second half tended to be "poorer" eggs. There appears to take place two cycles of egg maturing; the first reaches its peak about the middle of July, the second, I am informed, in late August or early September.

It would be of much interest to determine the exact physiologic condition of the eggs and of the sperm at the moment of natural shedding, throughout the breeding season. Vernon's observations ('99) need to be checked by more refined quantitative methods.

Between the first half of July and late August there appears to be a period during which the mature eggs deteriorate rapidly, and few if any unripe eggs mature. The behavior of the eggs in this latter period depends upon the relative numbers of unripe, ripe, and overripe eggs and the degree of overripeness. As these factors seem not to have been taken into account (Gemmill, '00) much confusion has resulted.

The agglutination test is in accord with other tests, all of which force one to conclude that "ovary" eggs show all gradations to extremely overripe eggs.

Other Sources of Variability.

When a comparison was made between the eggs tested immediately after receiving the *Arbacia* from the collecting boat, and eggs from the same group of *Arbacia* kept in tanks of running sea water 3 or more days, the eggs from the latter were more deteriorated. Even when the *Arbacia* were kept several days in the large floats at the wharf in the harbor, the eggs were more deteriorated than those eggs freshly tested upon receipt from the collecting boats. When the *Arbacia* were exposed to the sun during the trip to the laboratory, *i.e.*, when large numbers were kept in pails with insufficient sea water or exposed to the sun, such *Arbacia* gave a preponderance of deteriorated eggs.

When individual *Arbacia* were kept in jars containing 1,000 cc. to two gallons of sea water, changed twice daily, and the jars

placed in running sea water, the germ cells spontaneously shed from the intact animals. These germ cells were tested. The tests were size, color, shape of egg, rate of membrane formation, rate of cleavage and agglutination time. Many such spontaneously shed germ cells were in excellent physiologic condition. *Yet not infrequently such eggs showed surprising degrees of degeneration.* Such degenerate eggs were shed late, while the less degenerate eggs were shed early in the egg cycle. I attribute such degeneration to delayed shedding of eggs.

It was also noted that for several days after severe and protracted storms, *Arbacia* freshly collected and freshly tested gave germ cells in a deteriorated condition. It would seem as though severe storms or other adverse condition delays the natural shedding of germ cells with a consequent degeneration, the extent of degeneration being a function of the time that ripe eggs are retained within the body, and a function of the temperature of the sea water.

Extrusion of eggs, whether spontaneously or after opening the body, gives no assurance that the eggs are recently matured, *i.e.*, in good condition.

My evidence for "shed" versus "testes" sperm is not sufficient to draw any definite conclusions.

Summary.

1. *Arbacia* were freshly collected, freshly opened, the "best" eggs selected and immediately tested for agglutination time. The technique gave for duplicate tests, or for tests of aliquot portions of eggs, a difference in agglutination time of 0 to 4 seconds with an average difference of 1 second or 4.5 per cent. This is the experimental error.

2. (*a*) Eggs from different females when tested separately, under strictly comparable conditions, by the same sperm suspension from a single male, gave extremely wide differences in agglutination time, namely, 2 to 55 seconds or 9 to 1,300 per cent. Eggs that gave high agglutination values with one sperm gave consistently high, though not the same values with sperm from other males.

(*b*) These variations in agglutination time corresponded with

the variations in size, color and shape of eggs, loss of jelly, rate and per cent. of membrane formation, rate and per cent. of cleavage. All of them measure degrees of deterioration or overripening prior to shedding. Hence agglutination time may be used as another quantitative measure of deterioration of eggs.

(*c*) "Shed" eggs gave lower agglutination values and are less variable than "ovary" eggs, as defined, due to larger number of overripe eggs in the "ovary eggs."

(*d*) Severe storms and other adverse conditions that delay spontaneous shedding tend to deteriorate the eggs within the body, with corresponding changes in agglutination values.

3. (*a*) Suspensions of sperm from different males, in the same concentration, tested with the same egg water also gave a surprising amount of variation. They varied from 11 to 3,300 per cent.

(*b*) Sperm which gave high agglutination values with the eggs of one female gave consistently high, though not the same, values with eggs of other females. Sperm with low agglutination values gave low values with other females.

4. These large differences in agglutinability of different freshly shed sperms are due to corresponding physiologic deterioration or overripening prior to shedding.

5. The large differences in freshly shed eggs from different females is in small part due to genetic differences in agglutinin production, in largest part to deterioration of eggs within the body, prior to shedding.

6. Chronologically fresh, *i.e.*, "normal" germ cells may range from physiologically fresh to extremely overripe germ cells.

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